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In re Application of:
RADEMACHER et al.

Serial No. 10/009,629

Filed: December 12, 2001

Group Art Unit: 1616

Examiner: Pryor, Alton Nathaniel

For : Method of generating plants with an increased content of flavonoids and phenolic constituents

DECLARATION

1. I, Wilhelm Rademacher, Dr. rer. nat., a citizen of the Federal Republic of Germany and residing at Austraße 1, 67117 Limburgerhof, Germany, hereby declare as follows:

I am a fully trained Biologist having studied Biology at the University of Goettingen, Germany, from 1968 to 1973. I received a Diploma Degree in July 1973 by the University of Goettingen, Germany. In 1978, I received the doctorate degree (Ph.D.) by the University of Goettingen, Germany.

I joined BASF Aktiengesellschaft, 67056 Ludwigshafen, Germany, in 1980. Since then, I have been working in the field of crop protection. I have read and fully understood US application Ser. No. 10/009,629 and I am familiar with the subject-matter disclosed and claimed therein;

2. I have read and fully understood the Office Action of December 14, 2006 and the references cited therein by the Examiner;

3. The following observations are made by me.

4. Supplementary Experimental Data

4.1 Flowering hop plants (variety Willamette) were treated with a wide range of rates of prohexadione-Ca (applied as Apogee, a commercial formulation containing 27.5% prohexadione-Ca). Quercetin-3-O-glucoside (= quercitrin), a major hop flavonoid, was extracted and analyzed by standard methods 48 hours after treatment. Treated plants displayed a decrease of 79-80% in quercetin 3-glucoside content. Saturating effects were achieved with dosages of 50 ppm of prohexadione-Ca applied next to run-off.

These results are in line with data obtained in young apple shoots, in which significant reductions in quercitrin and the related isoquercitrin, rutin, and hyperin were detected after treatment with prohexadione-Ca (Roemmelt et al., Phytochemistry 64, 2003, 709-716, Fig. 6). Likewise, it can be expected that, instead of regular flavonoids, 3-deoxy equivalents are also formed in hop cones in the presence of prohexadione-Ca.

5. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1101 of Title 18 of the US-code and that such willful false statements may jeopardize the validity of the above-identified patent issued thereon.

Limburgerhof, June 11, 2007

(Wilhelm Rademacher)

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Formation of novel flavonoids in apple (*Malus×domestica*) treated with the 2-oxoglutarate-dependent dioxygenase inhibitor prohexadione-Ca

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Abstract

Novel flavonoids were formed in young leaves of apple (*Malus×domestica*) after treatment with the dioxygenase inhibitor prohexadione-Ca, which is known to reduce the incidence and severity of fire blight caused by *Erwinia amylovora* and other plant diseases. The compounds were isolated and identified as luteoliflavan, luteoliflavan 5-glucoside, eriodictyol 7-glucoside and 6″-*O*-*trans*-*p*-coumaroyleriodictyol 3′-glucoside. These flavonoids represent a novel biosynthetic pathway in apple leading to the formation of 3-deoxyflavans. Concomitantly, the content of regularly occurring phenylpropanoids is also influenced by prohexadione-Ca with increasing amounts of hydroxycinnamic acids and decreasing flavan-3-ols and flavonols. The altered flavonoid metabolism may be related to the lowered pathogen incidence though the isolated novel flavonoids do not exhibit antibacterial activity.

Keywords: *Malus×domestica*; Rosaceae; Apple, *Erwinia amylovora*; Resistance; Flavonoids; 3-Deoxyflavonoids; Dioxygenase inhibitor; Prohexadione-Ca

1. Introduction

Prohexadione-Ca is a recently developed plant growth retardant used in apple and other fruit trees (Evans et al., 1997; Byers and Yoder, 1999; Greene, 1999; Owens and Stover, 1999; Unrath, 1999; Basak and Rademacher, 2000). The compound acts as a structural mimic of 2-oxoglutarate thereby inhibiting dioxygenases, which catalyse distinct steps in the biosynthesis of the growth hormone gibberellin (Rademacher, 2000). As a result, longitudinal shoot growth is reduced. Several studies have revealed that application of prohexadione-Ca in addition reduces the incidence and severity of fire blight (caused by the bacterium *Erwinia amylovora*) (Fernando and Jones, 1999; Momol et al., 1999; Yoder et al., 1999; Costa et al., 2001), although the compound does not possess any bactericidal activities (Rademacher et al., 1999). Moreover, high dosage of prohexadione-Ca inhibits the formation of anthocyanins (Rademacher et al., 1992). This is possibly due to the fact that 2-oxoglutarate-dependent dioxygenases also play a key role in the biosynthesis of anthocyanins and other flavonoids (Heller and Forkmann, 1994). One of the possible target enzymes is flavanone 3-hydroxylase (FHT), which catalyses the conversion of flavanones to dihydroflavonols. As a result, metabolites would be channelled via an alternative flavonoid pathway to 3-deoxyflavans. So far, there has been no report about the occurrence of 3-deoxyflavonoids in *Malus×domestica*.

2. Results and discussion

Treatment of apple saplings with prohexadione-Ca, which is known to induce resistance against fire blight, led to the formation of novel compounds in the leaf tissue. Compound 1 reacted with *p*-dimethyl-aminocinnamaldehyde (DMACA), which is reported to be selective for flavans and particularly sensitive for flavanols (Treutter, 1989). Its UV absorbance (Table 1, Fig. 1)

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also indicated a flavanol structure. The high CRD/UV ratio of 36.6, which is even higher than the ratios found for catechin and epicatechin (Treutter et al., 1994) led to the assumption of a monomeric compound, which was further indicated by the relatively high R_f value after TLC separation (Table 1). The rather high retention time in HPLC indicated a reduced number of hydroxyl groups as compared to catechin and epicatechin. The compound was finally identified by NMR spectroscopy as the 3-deoxyflavonoid luteoliflavan (Table 2, Fig. 2). 3-Deoxyflavans have not been described to occur in apple so far (Treutter, 2001). The main feature is the absence of a hydroxyl group in position 3 at the C-ring as compared to catechin.

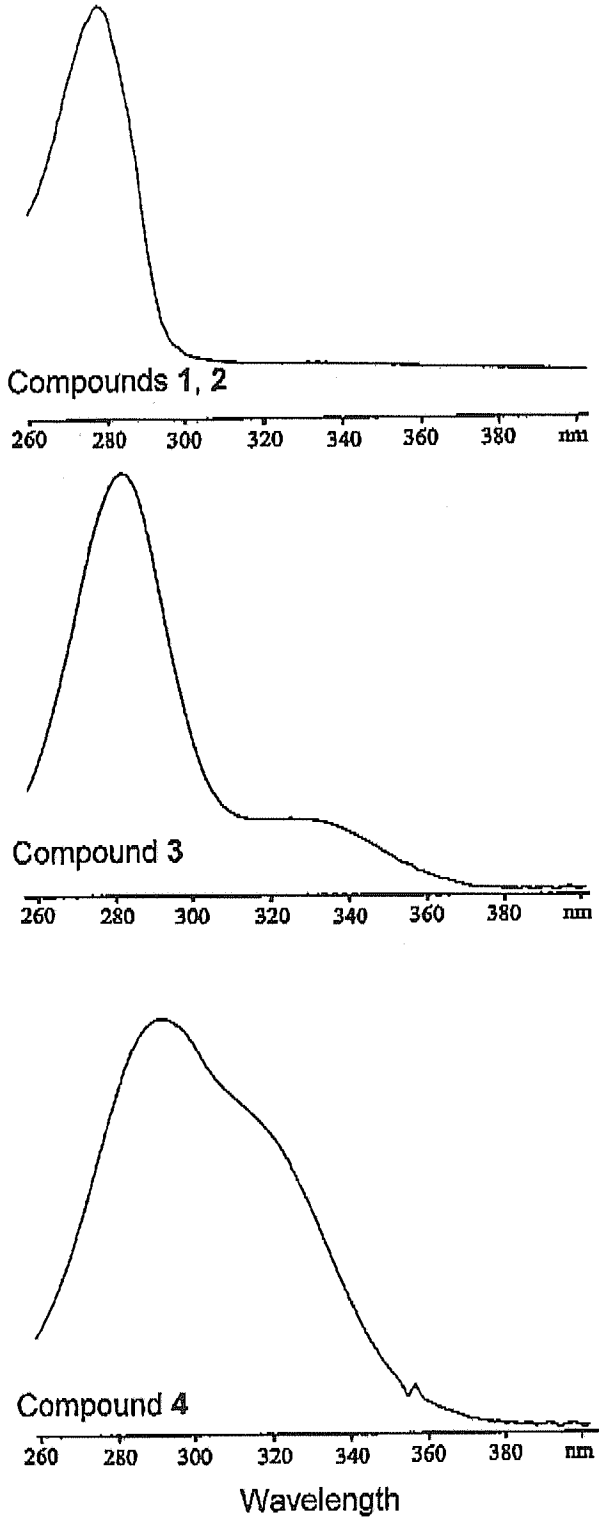

Fig. 1. UV absorption spectra of the compounds studied obtained by a diode array detector during an HPLC run.

The second compound (2) induced by prohexadione-Ca also reacted with DMACA . It showed a UV spectrum similar to that of luteoliflavan (Fig. 1). After enzymatic hydrolysis with β-glucosidase, luteoliflavan could be detected by TLC and HPLC. The structure was unequivocally identified as luteoliflavan 5-glucoside by NMR spectroscopy (Table 2, Fig. 2). The position of glycosylation is as in phloridzin indicating that the same glycosyltransferase might be involved in the conjugation of the aglycone luteoliflavan. No oligomeric proanthocyanidins possessing a luteoliflavan moiety were found in the tissue extracts, although procyanidins still occurred. The UV spectra of the third new compound (3) indicated the presence of a flavanone structure (Table 1). It co-migrated with authentic eriodictyol 7-glucoside in HPLC as well as TLC. Both acid and enzymatic hydrolyses revealed eriodictyol as the aglycone. The structure was further confirmed by NMR-spectroscopy (Table 2). Enzymatic hydrolysis of compound 4 using tannase gave *p*-coumaric acid and eriodictyol and it was identified as 6″-*O*-*trans*-*p*-coumaroyleriodictyol 3′-glucoside by NMR-spectroscopy (Table 2, Fig. 2).

Formation of these novel flavonoids in apple tissue indicates that prohexadione-Ca inhibits the 2-oxoglutarate dependent dioxygenase FHT, which catalyses the conversion of flavanones to dihydroflavonols (Heller and Forkmann, 1994). Inhibition of 2-oxoglutarate-dependent dioxygenases by prohexadione-Ca is well documented (Rademacher, 2000). The proposed enzyme inhibition results in a modified channelling of intermediates towards luteoliflavan and other compounds (Figs. 3, 7), which may be involved in defence reactions of apple. The question arises whether dihydroflavonol 4-reductase (DFR) is catalysing the formation of the

Table 1
UV spectral and chromatographic data of the compounds studies

Compound	UV absorbance λ_{max} (nm)	Retention time (min) (HPLC[a])	R_f value (TLC)
1	278	105.1	69.2[b]
2	61.5	61.5	25.3[b]
3	282, 324 (sh)	92.8	59.0[c]
4	292, 308 (sh)	171.9	

sh = shoulder.
[a] Column: 250×4 mm I.D., packed with Hypersil ODS 3 μm, solvents: HCO_2H (5% in H_2O), MeOH.
[b] Silica gel plates, solvents: toluene, Me_2CO, HCO_2H.
[c] Cellulose plates, solvents: *n*-BuOH, HOAc, H_2O.

flavan-4-ol (luteoforol) as the precursor of luteoliflavan using eriodictyol as substrate or whether flavanone 4-reductase (FNR) occurs in apple tissue. Recent work has shown, that the DFR of apple possesses also FNR activity and is, therefore, able to perform the key reaction in the formation of the 3-deoxyflavans (Fischer et al., 2003). Furthermore, the constitutive flavanone naringenin 7-glucoside accumulated to a higher level after treatment with prohexadione-Ca (Fig. 4). The absence of the aglycones eriodictyol and naringenin can be explained by a rapid conjugation of these compounds. It can be speculated that the glycosylation of the flavanones followed by acylation represents a detoxification mechanism by the plant cell.

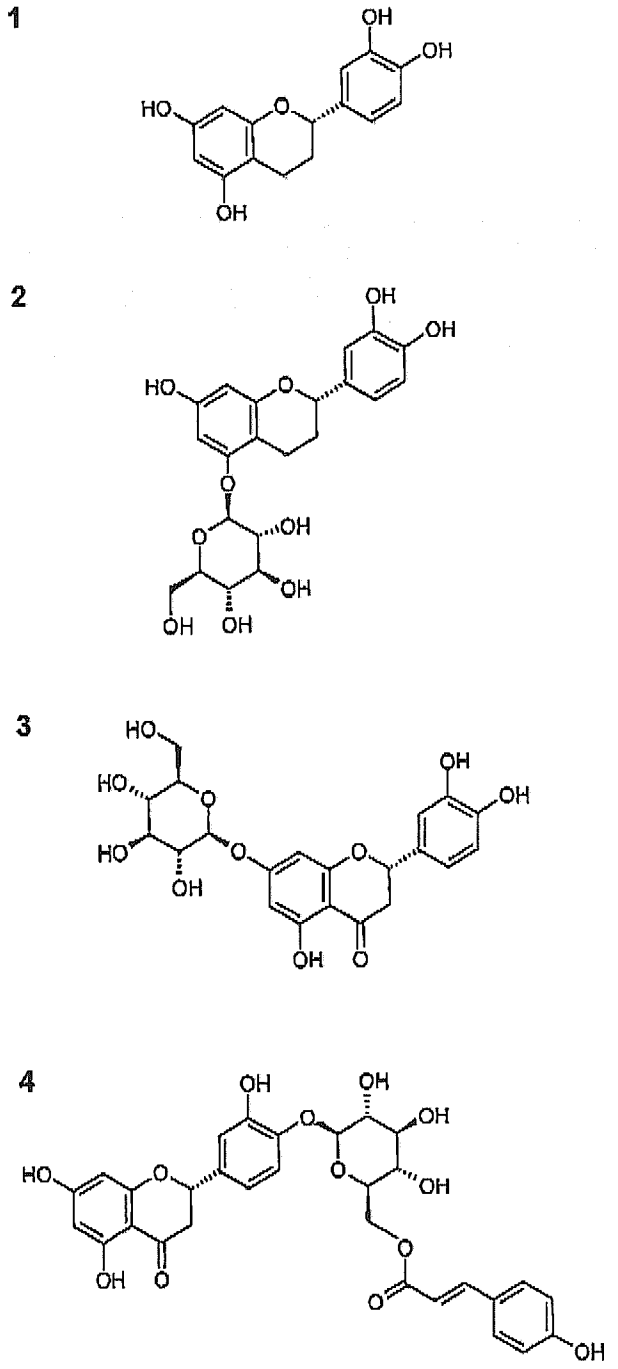

Fig. 2. Chemical structures of the compounds occurring in apple tissues after prohexadione-Ca treatment.

The pattern of constitutive compounds was also markedly affected by prohexadione-Ca. The concentrations of *p*-coumaric acid and chlorogenic acid were enhanced in treated leaves (Fig. 4). One may assume that the proposed inhibition of the flavonoid pathway at the level of FHT provokes a bottleneck for metabolites and, therefore, leads to the accumulation of cinnamic acids. Prohexadione-Ca markedly decreased the content of the oligomeric flavan-3-ol procyanidin B5 and more drastically E-B5 (Fig. 5). The flavonols hyperin, isoquercitrin, rutin and quercitrin were also dramatically reduced throughout the experiment (Fig. 6). The decrease of flavonols and flavan-3-ols clearly reflects the inhibition of FHT by prohexadione-Ca. Furthermore, it can be expected that flavonol synthase (FLS), another 2-oxoglutarate dependent dioxygenase, is also inhibited by prohexadione-Ca.

Luteoliflavan and the other flavonoids occurring upon prohexadione-Ca treatment do not show any remarkable effect on bacterial growth (Roemmelt et al.,

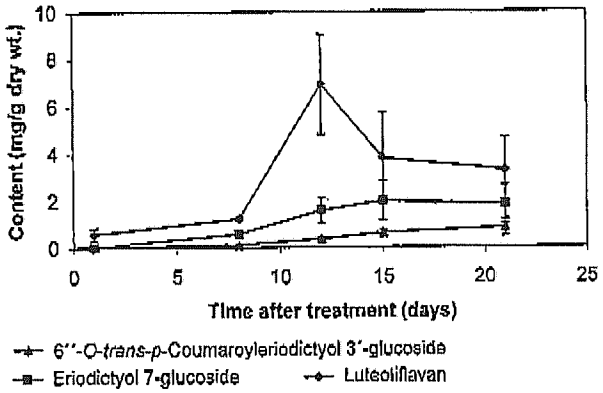

Fig. 3. Accumulation of flavonoids induced by prohexadione-Ca.

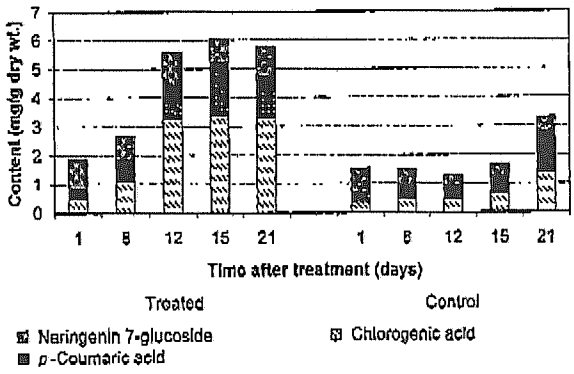

Fig. 4. Time course of naringenin 7-glucoside, *p*-coumaric acid and chlorogenic acid contents after treatment with prohexadione-Ca.

Table 2
^{1}H- and ^{13}C-NMR spectral data of the purified compounds (1H: 600 MHz, ^{13}C: 150 MHz, DMSO-d_6

Position	Compound 1		Compound 2		Compound 3		Compound 4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δH	δ_C	δ_H
C-2	80.0	4.50 (*d*)	76.4	4.75 (*dd*)	78.6	5.43 (*d*)	77.6	5.38 (*d*)
3	66.2	3.80 (*m*)	28.5	2.0 (*m*), 1.8 (*m*)	42.1	3.28 (*m*), 2.73 (*m*)	41.6	2.71 (*m*), 3.19 (*m*)
4	28.0	2.70 (*m*), 2.35 (*m*)	18.5	2.78 (*m*), 2.50 (*m*)	197.1		195.9	
5	157.1		156.4		162.6		163.4	
6	94.8	5.90 (*s*)	94.8	6.10 (*s*)	96.2	6.13 (*s*)	95.9	5.80 (*s*)
7	156.2		156.4		165.0		166.0	
8	94.0	5.70 (*s*)	96.5	5.85 (*s*)	95.2	6.14 (*s*)	95.2	5.84 (*s*)
9	156.2		156.1		162.6		162.7	
10	99.0		102.5		103.0		101.2	
1′	130.8		132.4		129.1		129.2	
2′	114.4	6.75 (*s*)	113.4	6.78 (*s*)	114.2	6.88 (*s*)	114.4	7.19 (*s*)
3′	145.8		145.3		144.4		144.6	
4′	145.7		144.9		144.4		146.6	
5′	115.0	6.70 (*d*)	115.0	6.68 (*d*)	115.2	6.74 (*d*)	115.8	6.84 (*d*)
6′	118.0	6.60 (*d*)	116.9	6.62 (*d*)	117.9	6.75 (*d*)	120.9	7.00 (*d*)
1″			100.5	4.70 (*d*)	99.3	4.97 (*d*)	101.3	4.38 (*d*)
2″			73.1	3.19 (*m*)	72.8	3.20 (*m*)	72.7	3.35 (*m*)
3″			76.6	3.22 (*m*)	76.1	3.26 (*m*)	75.1	3.33 (*m*)
4″			69.4	3.18 (*m*)	69.2	3.13 (*m*)	69.4	3.27 (*m*)
5″			76.8	3.22 (*m*)	76.9	3.37 (*m*)	73.7	3.70 (*m*)
6″			60.3	3.45 (*m*), 3.65 (*m*)	60.4	3.66 (*m*), 3.43 (*m*)	63.1	4.40 (*m*), 4.23 (*m*)
CO							166.5	
C-α							113.0	6.34 (*d*)
C-β							114.0	7.52 (*d*)
1‴							124.7	
2‴							130.0	7.45 (*d*)
3‴							115.2	6.73 (*d*)
4‴							159.7	
5‴							115.2	6.73 (*d*)
6‴							130.0	7.45 (*d*)

2002). If these flavonoids are not active against bacteria there obviously must exist other resistance mechanisms. It may be speculated that lignification or further metabolites, which are not detected by the methods used, are involved in defence.

3. Experimental

3.1. General

HPLC analysis was performed on a column (250×4 mm I.D.) prepacked with Hypersil ODS, 3 μm particle size, following a stepwise gradient using mixtures of HCO_2H (5% in H_2O) and MeOH from 95:5 (v/v) to 10:90 (v/v) with a flow rate of 0.5 ml/min. Hydroxycinnamic acids, flavonols and flavanones were detected at 280 nm whereas flavan-3-ols and 3-deoxyflavans were estimated at 640 nm after post column derivatisation with *p*-dimethyl-aminocinnamaldehyde (DMACA) (Treutter, 1989). The constitutive phenolic compounds have been previously described in apple (Mayr et al., 1995) and were identified by comparison with authentic samples using a diode array detector for spectra comparison. In addition naringenin 7-glucoside (prunin) could be identified (Rt 116.9, λ_{max} 282 nm).

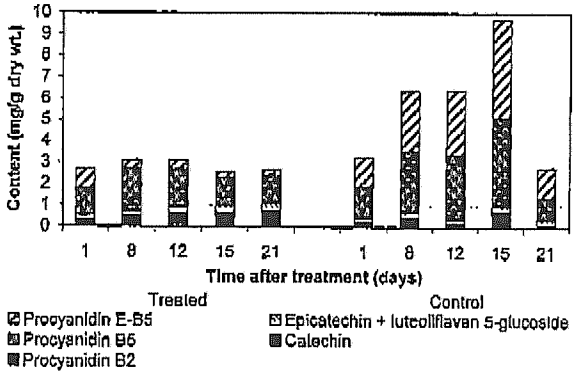

Fig. 5. Time course of flavan-3-ol contents after treatment with prohexadione-Ca.

3.2. Plant material

Four year old trees of *Malus×domestica* cv. M9 cultivated in a greenhouse were sprayed with the dioxygenase inhibitor prohexadione- Ca at a concentration of 250 ppm active ingredient. The formulation BAS 125 10 W (Regalis®), a wettable granular containing 10% by weight of prohexadione-Ca was used. The three youngest not fully developed leaves per shoot were sampled two weeks after treatment. The leaves were frozen in liquid nitrogen immediately after sampling and afterwards lyophilized.

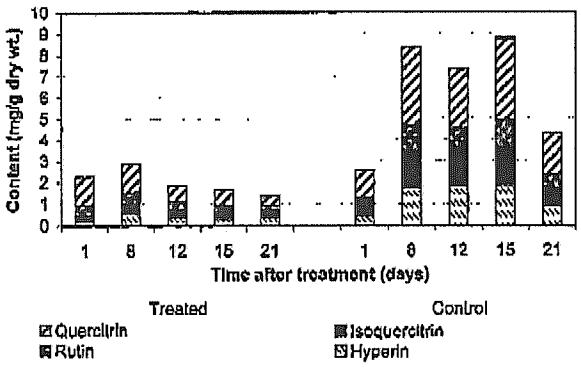

Fig. 6. Time course of flavonol contents after treatment with prohexadione-Ca.

3.3. Extraction and isolation

Methods for purification of the unknown compounds of *Malus×domestica* were elaborated. The lyophilized apple leaves (500 g dry weight) were pulverised in an Ultraturrax and extracted exhaustively with MeOH. The combined extracts were evaporated in vacuo to 1.2 l, diluted with H_2O (4.8 l), and extracted in portions of 200 ml, each, eight times with petrol, boiling range 60–80 °C (200 l in total). Phenolic compounds were extracted five times with EtOAc (120 l in total) from the aqueous phase. The combined EtOAc extracts were evaporated to dryness, resuspended in water and lyophilized yielding a residue of 70 g. The dry residue was dissolved in 220 ml EtOH, divided into three portions and subjected to chromatography on three glass columns (2.5 cm diameter ×35 cm length) packed with Sephadex LH 20. The phenolic compounds were eluted with 1.5 l EtOH and collected in portions of 25 ml, which were analysed for phenolic compounds by TLC and HPLC and combined to two crude portions between 150 and 250 ml elution volume (portion II) and between 250 and 450 ml elution volume (portion I), which were further purified:

Portion I was concentrated to a small volume, divided into two portions and rechromatographed on two further Sephadex LH 20 columns as described above.

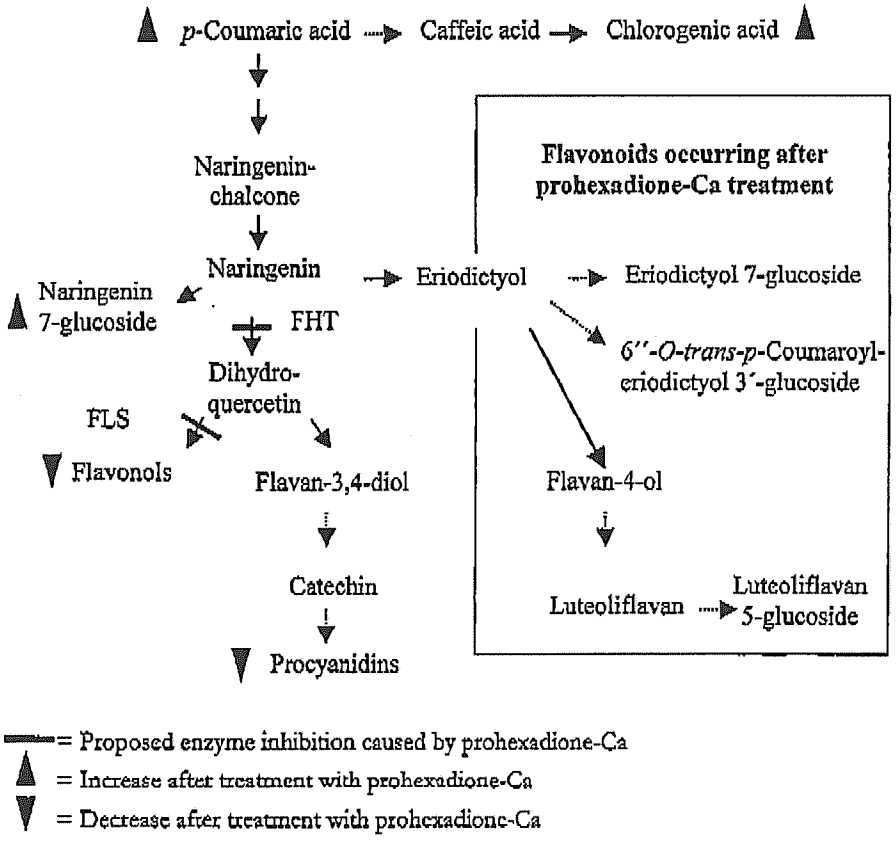

Fig. 7. Flavonoid metabolism of apple leaves. A channelling of the pathway towards 3-deoxyflavans is observed by the prohexadione-Ca treatment. FHT = flavanone 3-hydroxylase; FLS = flavonol synthase.

Fractionation was performed as described above and the compound of interest eluted between volume 210 and 520 ml. The solvent was evaporated, the residue redissolved in water and the solution was stored at 4 °C for a few hours resulting in precipitation of phloridzin, the main phenolic component in apple leaves, and several other compounds. It was centrifuged and a part of the supernatant was further purified by semi preparative HPLC (column: 2.5 cm diameter, 25 cm length, packed with Polygosil ODS 5 μm) with H_2O (A) and MeOH, gradient grade, (B) as solvents, using the following gradient: 5–10% B in A: 0–1000 ml, 10–15% B in A: 1000–2000 ml, 15% B in A: 2000–3000 ml, flow rate: 6 ml/min. The 15% B fraction was evaporated, redissolved in water and rechromatographed on a Polyamide column (2 cm diameter×8.5 cm length) using H_2O (A) and MeOH, gradient grade, (B) as solvents with a gradient from 30 to 40% B in A yielding 0.6 mg of compound 1 in the 40% B fraction.

Portion II was lyophilized (45 g dry weight), resuspended in water and subjected to chromatography on a glass column (2.5 cm diameter×35 cm length) packed with Sephadex LH 20 and eluted with a H_2O-MeOH gradient ranging from 0 to 70% MeOH in 10% steps with a volume of 250 ml each. The collected fractions (IIa, IIb, IIc) were further purified by several semi preparative HPLC runs (column 2.5 cm diameter, 25 cm length, packed with Polygosil ODS 5 μm) using H_2O (A) and MeOH, gradient grade, (B) as solvents following different stepwise gradients: *Fraction IIa* (40% MeOH): 5% B in A, isocratically, 120 min; 5–10% B in A, 60 min; 10% B in A, isocratically, 120 min; 10–15% B in A, 60 min; 15% B in A, isocratically, 60 min; 15-20% B in A, 60 min; 20% B in A, isocratically, 180 min; flow rate: 5 ml/min. The fraction at 20% MeOH (2458–2841 ml) contained 4.1 mg of compound 3. *Fraction IIb* (50% MeOH): 10% B in A, isocratically, 140 min; 10-15% B in A, 120 min; 15% B in A, isocratically, 340 min; flow rate: 5 ml/min. The fraction 10–15% MeOH contained compound 2, which eluted together with epicatechin. It was purified by further semi-preparative HPLC runs, using the following gradient: 0% B in A, isocratically, 450 min; 0–5% B in A, 120 min; 5% B in A, isocratically, 340 min; 5–10% B in A, 120 min; 10% B in A, isocratically, 350 min; flow rate: 5 ml/min: The fraction at 10% MeOH (6160–6700 ml) contained 4.7 mg of compound 2. *Fraction IIc* (70% MeOH): 30% B in A, isocratically, 225 min; 30–35% B in A, 20 min; 35% B in A, isocratically, 125 min; 35–40% B in A, 20 min; 40% B in A, isocratically, 90 min; 40–45% B in A, 20 min; 45% B in A, isocratically, 410 min; 45–50% B in A, 20 min; 50% B in A, isocratically, 200 min; 50–55% B in A, 20 min; 55%, isocratically, 60 min; 55–60% B in A, 20 min; 60% B in A, isocratically, 50 min; flow rate: 4 ml/min. The fractions at 45% MeOH were purified by further semi preparative HPLC runs using following gradient: 35% B in A, isocratically, 120 min; 35–40% B in A, 60 min; 40% B in A, isocratically, 120 min; 40–45% B in A, 60 min; flow rate: 4 ml/min. The fraction between 40 and 45% MeOH (1440–1560 ml) contained 3.1 mg of compound 4.

3.4. Hydrolysis of phenolic glycosides

For enzymatic hydrolysis the purified compounds were evaporated and redissolved in NaOAc-buffer (pH 4.6, 0.1 M). β-Glucosidase from almonds (Sigma) or tannase (Braunschweiger Biotechnologie), respectively, were added and the samples were kept at 37 °C for 17 h. The enzymatic reaction was stopped with MeOH and the sample was extracted three times with EtOAc. The combined EtOAc extracts were evaporated to dryness and redissolved in MeOH for HPLC-analysis. The hydrolysis products were compared with authentic standards on HPLC and TLC.

3.5. TLC

Purified compounds and hydrolysis products were compared with standards on TLC plates. Compounds 1 and 2 were separated on silica gel plates developed with toluene:Me_2CO:HCO_2H (3:6:1, v/v/v). The spots were visualised by spraying with a solution of DMACA (1% in 6 M HCl:EtOH = 1:1, v/v). Compound 3 was separated on cellulose plates developed with *n*-BuOH: HOAc:H_2O (4:1:5, v/v/v). The spots were visualised by spraying a methanolic solution (1%) of natural product reagent A (Roth, Karlsruhe, Germany).

3.6. Molecular spectroscopy and structure elucidation

Purified phenolic compounds were identified by NMR spectroscopy, high-resolution mass spectrometry and structure comparisons using a BASF customized version of the SpecInfo Spectral database (Bremser, 1988; Bremser und Fachinger, 1985). All NMR experiments were performed on a Varian INOVA 600 NMR spectrometer equipped with an inverse 3 mm ^1H {^{13}C,^{15}N} gradient probe at room temperature. NMR samples were dissolved in 120 μl DMSO-d_6. The sample solutions were transferred into a Shigemi 3 mm NMR tube with susceptibility matched glass for DMSO. NMR data sets consisted of 1D-H NMR-, 2D-magic-angle-gradient-DQF-COSY- (Mattiello et al., 1996), multiplicity edited 2D-^1H,^{13}C-gradient-HSQC- (Schleucher et al., 1994) and 2D-^1H,^{13}C-gradient HMBC (Bax and Summers, 1986; Vuister et al., 1991) spectra.

All mass spectra were recorded on a Finnigan Time-of-flight instrument in high resolution mode equipped with an electrospray ion source. The assignment strategy started with the identification of directly bound CH_x-units from HSQC spectra. The applied multiplicity

filter within the ^{13}C-evolution period of the HSQC data helped to distinguish CH and CH_3 groups from the opposite phased CH_2 groups. These CH-building blocks were subsequently assembled using CH-long range correlations from the HMBC experiment as well as proton spin systems from COSY correlations. Remaining ambiguities were resolved on the first hand by validation of the ^{13}C NMR shifts within the SpecInfo database. Finally, the results were matched with a list of the most probable molecular formulae calculated from the high-resolution mass spectra. Similar structures from the Beilstein and Chapman and Hall compound databases were used as well to check the validity of the assignments.

3.7. Time course study

One year old trees of *Malus×domestica* cv. M9 cultivated in a growth chamber were sprayed with prohexadione-Ca at an average new shoot length of 10 cm as described above. Control plants were sprayed with water. For HPLC analyses of phenolic compounds the youngest, fully unfolded leaf per shoot was collected 1, 8, 12, 15 and 21 days after treatment and immediately frozen in liquid nitrogen. After lyophilization the samples were ground and extracted with methanol containing 6-methoxyflavone as an internal standard. The extract was centrifuged, the solvent evaporated and the residue redissolved in a small volume of MeOH. The extracts were directly used for HPLC-analysis. Three leaves of three different plants as replicates per treatment and sampling date were analyzed separately.

Acknowledgements

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